Disinhibitory Recruitment of NMDA Receptor Pathways in Retina

Running Head: Regulation of NMDAR activation in RGCs

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ABSTRACT

Glutamate release at bipolar to ganglion cell synapses activates NMDA and AMPA/KA ionotropic glutamate receptors. Their relative strength determines the output signals of the retina. We found that this balance is tightly regulated by presynaptic inhibition that preferentially suppresses NMDA receptor (NMDAR) activation. In transient ON-OFF neurons, block of GABA and glycine feedback enhanced total NMDAR charge by 35-fold in the ON response, 9-fold in the OFF compared to a 1.7-fold enhancement of AMPA/KA receptors. Blocking only glycine receptors (GlyRs) enhanced the NMDAR EPSC 10-fold in the ON and 2-fold in the OFF pathway. Blocking GABA\textsubscript{A} or GABA\textsubscript{C} receptors produced small changes in total NMDAR charge. When both GABA\textsubscript{A} and GABA\textsubscript{C} receptors (GABA\textsubscript{A&C}Rs) were blocked the total NMDAR charge increased 9-fold in the ON and 5-fold in the OFF pathway. This exposed a strong GABA\textsubscript{C}R feedback to bipolar cells that was suppressed by serial amacrine cell synapses mediated by GABA\textsubscript{ARs}. The results indicate that NMDAR currents are large but latent, held in check by dual GABA and glycine presynaptic inhibition. One example of this controlled NMDAR activation is the crosstalk between ON and OFF pathways. Blocking the ON pathway increased NMDAR relative strength in the OFF pathway. Stimulus prolongation similarly increased the NMDAR relative strength in the OFF response. This NMDAR enhancement was produced by a diminution in GABA and glycine feedback. Thus, the retinal network recruits NMDAR pathways through presynaptic disinhibition.
INTRODUCTION

Ionotropic glutamate receptors are of two types, NMDARs and non-NMDARs (AMPA/KARs). Retinal ganglion cells (RGCs), the output neurons of the retina, receive excitatory glutamatergic input from bipolar cell terminals and express both AMPA/KARs and NMDARs (Bloomfield and Dowling 1985; Gottesman and Miller 1992; Lukasiewicz and McReynolds 1985; Slaughter and Miller 1983). NMDARs are characterized by higher glutamate affinity and slower kinetics (Bekkers and Stevens 1989; Chen and Diamond 2002; Jahr and Stevens 1987; McBain and Dingledine 1992; Mittman et al. 1990; Patneau and Mayer 1990). Hence NMDARs and AMPA/KARs are thought to be complementary, extending the response range of the RGCs to read the output of bipolar cells (Buldyrev et al. 2012; Manookin et al. 2010). NMDARs and AMPA/KARs can combine to provide a linear correlation between excitatory synaptic input and spiking behavior over a range of stimulus strengths (Diamond and Copenhagen 1995). It has been suggested that the relative activation of NMDARs and AMPA/KARs also induce variations in response kinetics and contrast sensitivities in specific cell types (Sagdullaev et al. 2006).

Studies determining the relative strength of NMDAR to AMPA/KAR activation during ganglion cell synaptic stimulation have been conflicting. Some indicate that blocking NMDARs has a small effect on the light evoked synaptic activity, even though functional NMDARs are expressed (Cohen and Miller 1994; Coleman and Miller 1988; 1989; Massey and Miller 1988; 1990; Slaughter and Miller 1983). Other studies indicate that NMDARs produce a significant component of the RGC synaptic responses to light, electrical and pharmacological stimuli (Buldyrev et al. 2012; Chen and Diamond 2002; Diamond and Copenhagen 1993; 1995; Kalbaugh et al. 2009; Manookin et al. 2010; Matsui et al. 1998; Mittman et al. 1990; Sagdullaev et al. 2011; Sagdullaev et al. 2006; Taylor et al. 1995). It is possible that these reports are from different cell types with dissimilar expression of NMDARs accounting for the variable activation of NMDARs (Manookin et al. 2010). However, it is also likely that the differences are an outcome of strong regulation of NMDAR activation (Matsui et al. 2001; Sagdullaev et al. 2006). This work aimed to address that possibility and to determine the degree and mechanisms involved. The results
indicate that synaptic input to ON-OFF ganglion cells can transition from AMPA/KA to NMDA receptor dominance under the control of presynaptic bipolar cell inhibition. We found that the crosstalk between ON and OFF pathways provides one example where this transition occurs in the normal operation of the retina.

METHODS

Tissue preparation: Larval tiger salamanders (*Ambystoma tigrinum*) were obtained from Charles Sullivan (Nashville, TN) and Kons Scientific (Germantown, WI) and were kept in tanks maintained at 4°C on a 12 h light/dark cycle. The animals were decapitated, and the eyes were enucleated. All procedures were performed in accordance with the US Animal Welfare Act and the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and were approved by the University Animal Care Committee at the State University of New York. The eyeballs were hemisected under infra-red light and the posterior eye cup was placed in oxygenated Ringer’s solution. The retina was detached from the pigment epithelium and flat mounted on a glass cover slip (Belleco Glass, Vineland, NJ) coated with poly l-lysine (Sigma-Aldrich Corp., St. Louis, MO) with ganglion cells facing up. For slices, the retina was flat mounted ganglion side up on a 0.22 µm pore membrane filters (Millipore, Bedford, MA) and sliced at 150-250 µm using a tissue slicer (Stoelting, Woods Dale, IL). Slices were rotated 90° and mounted on coverslips using vacuum grease (Dow Corning Corp., Midland, MI). All electrophysiological experiments were done under infrared light. Coverslips with either a whole mounted retina or a retinal slice were transferred to the recording chamber attached to an upright Zeiss Axioskop2 FS fluorescent microscope, equipped with a 40X Achromat water immersion objective. An infrared sensitive CCD camera (Hamamatsu, Japan) was used to capture the image of the preparation.

The tissue was constantly superfused with oxygenated Ringer’s solution containing (in mM): 111 NaCl, 2.5 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES and 10 dextrose buffered to pH 7.8 using NaOH. A gravity-fed perfusion system was used to maintain a flow rate of ~1.5 ml/min for control Ringer’s solution.
Electrophysiology: Recordings were made from neurons in the ganglion cell layer of both wholemounts and slices at room temperature. In wholemount retina, the glial end feet were removed using an 8-10 MΩ electrode filled with Ringer’s solution to expose the soma of ganglion cells. First, the exposed neurons were sampled for extracellular spike activity by a loose seal (25-50 MΩ) using an 8-10 MΩ electrode filled with Ringer’s solution. Based on the extracellular spike recordings, ON-OFF transient cells were identified then patched for whole cell recordings using a 5-7 MΩ electrode containing (in mM): 100 potassium gluconate, 5 NaCl, 1 MgCl₂, 5 HEPES, 5 EGTA buffered to pH 7.4 with KOH.

Data were acquired using a Multiclamp 700B Amplifier (Molecular Devices, Sunnyvale, CA). Analog signals were low-pass filtered at 2 kHz and sampled at 10 kHz with the Digidata 1322A analog-to-digital board (Molecular Devices). Clampex 10.1 software (Molecular Devices) was used to control the voltage command outputs, acquire data and trigger stimuli. The currents shown are raw data and were not corrected for electrode junction potential and access resistance. Both the series resistance and membrane capacitance were constantly monitored by a -20mV square pulse (50ms duration) before every light stimulus. Cells in which neither parameter changed during the entire course of the experiment were considered for further analysis. Drug solutions were delivered through a pressure fed Octaflow 2 perfusion system (ALA scientific instruments, Farmingdale, NY). Picrotoxin, strychnine, MFA (meclofenamic acid) and 18α-glycyrrhetinic acid were purchased from Sigma-Aldrich Corp. D-AP5 (D-2-amino-5 phosphonovaleric acid), L-AP4 (L-(-)-2-amino-4-phosphonobutyric acid), SR-95531 (6-imino-3-(4-methoxyphenyl)-1(6H)pyridazinethanoinic acid hydrobromide), TPMPA ((1,2,5,6-tetrahydropyridin4yl)methylphosphinic acid) and NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7 sulfonamide ) were obtained from Tocris Bioscience (Minneapolis, MN).

Light stimulation: Photoreceptors were stimulated by a 200 µm spot from a red light-emitting diode (LED, \( λ_{max} = 640 \, \text{nm} \)) projected through the objective lens. The irradiance between 620 and 660 nm of the LED was ~1.2 µW cm\(^{-2}\), measured by a RPS900-R wideband spectroradiometer (International Light, Peabody, MA). This light intensity has
been used to stimulate cones (Song and Slaughter 2010). A 1-second duration light stimulus was presented every 25 seconds.

Electrical stimulation: Bipolar cells in retinal slices were directly stimulated by short pulses (1 ms) of current delivered through an electrode filled with Ringer’s solution that was placed into the outer plexiform layer directly above the patched ganglion cell (Awatramani and Slaughter 2001). The pulses were generated with a constant-current stimulator (Grass S48 with stimulus isolation unit PSIU6, Grass Instruments, West Warwick, RI).

Data analysis: Traces were imported into IgorPro 6.22 (Wavemetrics, Inc.) for making figures and further analysis. Unless otherwise mentioned, the total charge transferred by the EPSC after light onset and offset was used as a measure of the ON and OFF light responses respectively. The pooled data were imported to Microsoft Excel 2007 to make graphs and for statistical tests. Pooled data are expressed as mean ± standard error. Student’s t-test was used to compare values under different conditions and was paired unless mentioned.

Differences were considered significant when $p \leq 0.05$.

RESULTS

**Presynaptic inhibition regulates the activation of NMDARs in ON-OFF transient cells.**

ON-OFF transient cells in the ganglion cell layer (GCL) were initially identified based on their light-evoked spike activity using a loose-patch recording. They were characterized by a short transient burst of spikes at both the onset and offset of light (Fig 1A inset). Once identified, whole cell patch electrodes were used to record light evoked transient ON-OFF EPSCs (L-EPSCs), representing glutamate output from bipolar cells (Fig 1A). The L-EPSCs were recorded at -70mV, close to the calculated chloride reversal potential ($E_{Cl} = -71$mV). At -70 mV, NMDARs are subject to magnesium block by the control Ringer’s solution (1 mM Mg$^{2+}$). However, removal of Mg$^{2+}$ from the Ringer’s solution (nominally Mg$^{2+}$ free) did not produce a statistically significant change in the L-EPSC (ON: 96 ± 13%, OFF: 107 ± 10%) (Fig 1A, B). A competitive NMDAR antagonist, D-AP5 (50 µM), in Mg$^{2+}$ free Ringer’s solution slightly reduced the L-EPSCs (ON: 92 ±
9% remaining, OFF: 83 ± 6%), only the change at OFF was statistically significant (Fig 1A, B). Overall, removing magnesium did not produce a significant enhancement and NMDAR block produced a relatively small suppression in synaptically driven light responses under our control experimental conditions. This is consistent with previous studies indicating that light evoked glutamatergic output from bipolar cells activates mainly AMPA/KARs (Coleman and Miller 1988; 1989; Slaughter and Miller 1983). However, when bipolar cell dendrites were electrically stimulated in slices, it activated both AMPA/KA and NMDA receptors. Mg$^{2+}$ free Ringer’s solution increased the electrically evoked EPSC (E-EPSC) compared to control (184 ± 20%) (Fig 1C, D). D-AP5 significantly reduced the enhanced E-EPSC (25 ± 10%, remaining) (Fig 1C, D). Hence NMDARs can be synaptically activated with strong stimulation but their activation by light stimulation is small under our control conditions. Furthermore, NMDARs are subject to Mg$^{2+}$ block but a Mg$^{2+}$ free Ringer’s solution had little effect on the network under our control conditions.

The possibility that weak activation of NMDARs was due to low concentrations of the co-agonist, either glycine or D-serine, was tested (Stevens et al. 2003). However, under control conditions the addition of 100 µM D-serine did not alter the light responses in ON-OFF cells (Data not shown).

When inhibition was blocked there was a marked increase in the L-EPSC total charge and a large increase in light-evoked synaptic NMDAR responses in ON-OFF cells, similar to E-EPSCs shown in figure 1. In retinas treated with 100 µM picrotoxin (PTX, which blocks GABA$\alpha_\text{Rs}$ and GABA$\gamma_\text{Rs}$ in amphibian retina) and 10 µM strychnine (STR, which blocks GlyRs) in Mg$^{2+}$ free Ringer’s solution, the L-EPSCs increased (ON: 407 ± 72%, OFF: 262 ± 34%) (Fig 2A, B). D-AP5 reduced the enhanced L-EPSCs (ON: 32 ± 5% remaining, OFF: 39 ± 5%) (Fig 2A, B). Overall, PTX and STR increased the ON EPSC by 407% and increased the percentage of the NMDAR component from 8% to 68% (Fig 2C). Therefore, the NMDAR EPSC charge increased about 35 fold. In the OFF pathway, PTX + STR increased the L-EPSC by 262% and the NMDAR fraction increased from 17% to 61%, the NMDAR charge increased by over 9 fold (Fig 2C). The AMPA/KAR EPSC in PTX and STR was not augmented as much (Fig 2 A, B). To evaluate this, D-AP5 was used
to isolate the non-NMDAR component of the L-EPSCs. PTX+STR increased the non-
NMDAR EPSC total charge by 1.7 fold in both ON and OFF pathways (ON: 1.72 ± 0.39,
OFF: 1.72 ± 0.29) (Fig 2 D, E). In summary, PTX and STR increased the non-NMDAR ON
and OFF responses by less than two fold while it increased the NMDAR responses by 35
fold in the ON and 9 fold in the OFF EPSC. Hence presynaptic inhibition
disproportionately suppressed the NMDAR component of L-EPSCs. In 4 cells, the
combination of 50 µM D-AP5 and 10 µM NBQX, a competitive AMPA/KAR antagonist,
blocked the PTX+STR enhanced L-EPSCs almost completely (ON: 5 ± 3% remaining,
OFF: 8 ± 4%) (Fig 2F), indicating that almost all the excitatory input to ganglion cells
could be eliminated by combining AMPA/KARs and NMDARs antagonists.

**Glycine inhibition plays a major role in the regulation of NMDAR activation**

Based on these findings, experiments were performed to determine if NMDAR
activation was preferentially regulated by GABAₐRs, GABAₖRs or GlyRs. The role of
glycine inhibition in regulating the activation of NMDARs was tested using 10 µM STR. In
6 cells, STR in Mg²⁺ free Ringer’s solution increased the L-EPSC in both ON and OFF
pathways (ON: 155 ± 18%, OFF: 134 ± 14%) (Fig 3A, B). D-AP5 reduced the STR-
enhanced L-EPSCs (ON: 49 ± 8% remaining, OFF: 72 ± 9%). Thus glycine inhibition
produced a 10-fold increase in the NMDAR charge in the ON pathway and a two-fold
increase in the OFF pathway. This is qualitatively similar to the effects of PTX + STR but
about a quarter of the magnitude.

In another 3 ON-OFF cells STR reduced the L-EPSCs (ON: 29 ± 4%, p < 0.005;
OFF: 47 ± 4%, p < 0.005) and D-AP5 had a small and statistically insignificant effect on
the STR L-EPSC (ON: 131 ± 14%, p = 0.167; OFF: 102 ± 26%, p = 0.95). This STR-
induced reduction of L-EPSCs is probably the result of serial inhibition (Zhang et al. 1997),
as described below.

The role of GABAₐ inhibition was tested using SR-95531 (SR, gabazine), a
selective GABAₐR antagonist. SR (10 µM) in Mg²⁺ free Ringer’s solution increased the
peak of the L-EPSC (ON: 224 ± 36%, OFF: 168 ± 20%) but also abbreviated the response
(Fig 3C, and inset in D). Only the total synaptic charge in the OFF response was enhanced
by SR (ON: 106 ± 18%; OFF: 192 ± 34%) (Fig 3D). In the ON pathway, SR enhanced the initial peak but suppressed the prolonged component, resulting in little change in the total synaptic charge at light onset. The reduction in the prolonged component (portion of the EPSC after the peak) probably results from unmasking other inhibitory circuits when GABA\(_A\)Rs are blocked. In the ON pathway, D-AP5 did not reduce the L-EPSC significantly (ON peak: 83 ± 8% remaining; ON area: 87 ± 9%) (Fig 3C, D). In the OFF pathway, where SR increased both the peak and total charge of the light response, D-AP5 reduced both (OFF peak: 73 ± 9% remaining; OFF area: 72 ± 9%) (Fig 3C, D, inset). The NMDAR component in the OFF pathway increased from 17% in control to 28% when GABA\(_A\)Rs was blocked, and there was a 3-fold increase in the total NMDAR charge.

Glycine and GABA\(_A\) pathways had similar effects in suppressing NMDAR circuits in the OFF pathway. GABA\(_A\)Rs had little effect in the total charge in the ON pathway, but interpretation was difficult because SR made the ON response more transient. When blocking an inhibitory pathway results in less excitation, it is likely that a serial inhibitory network is involved. Since GABA\(_C\)Rs are particularly important in feedback inhibition to ON bipolar cells, the antagonist TPMPA was used to explore the role of GABA\(_C\) inhibition in regulating the activation of NMDARs. TPMPA (100 µM) in Mg\(_{2+}\)-free Ringer’s solution increased the L-EPSC in both the ON and OFF pathways (ON: 136 ± 5% remaining, OFF: 125 ± 10%) (Fig 3E, F). D-AP5 had a small effect on the TPMPA-enhanced L-EPSCs in both the ON and OFF pathways (ON: 86 ± 8% remaining, OFF: 93 ± 8%) (Fig 3E, F). This would suggest that GABA\(_C\)R pathways play little role in the activation of NMDARs in both ON and OFF pathways. Since TPMPA alone did not reduce the L-EPSC in any of the cells, it seems likely that GABA\(_C\) inhibition does not regulate other inhibitory circuits (serial inhibition). This is consistent with studies indicating that GABA\(_C\)Rs are localized primarily to bipolar cell terminals (Lukasiewicz and Werblin 1994; Sagdullaev et al. 2006).

In summary, the NMDAR component in the ON pathway increased from 8% in control to 51% in STR, to 13% in SR and to 14% in TPMPA (Fig 3G). Only STR induced an ON NMDAR component comparable to PTX+STR. In the OFF pathway, the NMDAR component changed from 17% in control to 28% in STR, 27% in SR and 7% in TPMPA.
The OFF NMDAR component in the presence of PTX+STR (61%) was significantly higher.

**GABA$_A$ inhibition regulates activation of GABA$_C$ inhibition**

Unlike the data presented above, previous reports suggest that GABA$_C$Rs regulate the activation of NMDARs (Matsui et al. 2001; Sagdullaev et al. 2011; Sagdullaev et al. 2006). This could be a species difference as the other studies were performed in mouse retina. Another possible explanation is that our recordings were from retinal wholemounts while most of the data in these reports were from retinal slices (but see Sagdullaev et al. 2011). Such a discrepancy has been reported before in the effect of D-serine as a co-agonist for NMDAR activation. The co-agonist site seems to be saturated in retinal slices but not in wholemounts (Kalbaugh et al. 2009; Stevens et al. 2010). When we recorded from ON-OFF transient cells in the retinal slice preparation, 100 µM TPMPA in Mg$^{2+}$ free Ringer’s solution increased the L-EPSC in both ON and OFF pathways (ON: 148 ± 13%, OFF: 145 ± 10%) (Fig 4A, B). D-AP5 (50 µM) reduced the TPMPA-enhanced L-EPSC in both the ON and OFF pathways (ON: 51± 12% remaining, OFF: 39 ± 11%) (Fig 4A-C). This result implies that GABA$_A$R or GlyR circuits suppress activation of GABA$_C$R synapses in the wholemount retina preparation. These inhibitory circuits may be truncated in retinal slices, thereby disinhibiting GABA$_C$R pathways. Studies have shown that GABA$_A$R inhibition regulates activation of GABA$_C$Rs (Buldyrev and Taylor 2013; Eggers and Lukasiewicz 2006; Roska et al. 1998; Vigh et al. 2011; Zhang et al. 1997). This leads to the prediction that blocking GABA$_C$Rs along with GABA$_A$Rs would increase the NMDAR component of the L-EPSCs in wholemount retina.

TPMPA (100 µM) with SR (10 µM) in the wholemount retina increased the L-EPSCs in transient ON-OFF cells (ON: 187± 26%, OFF: 197 ± 33%) (Fig 4D, E). D-AP5 (50 µM) reduced the SR+TPMPA enhanced L-EPSCs (ON: 63± 13% remaining; OFF: 53 ± 7%) (Fig 4D, E). Thus SR+TPMPA produced a significant NMDAR component in the ON pathway (37%), which was not observed with either drug alone. Further, in the OFF pathway, SR+TPMPA produced an NMDAR component significantly higher than control (Fig 4F). Thus the combined effect of SR+TPMPA was more profound than summation of
their individual effects. This supports the premise of serial inhibition and that GABA_A inhibition suppresses GABA_C feedback to bipolar cells.

The combination of SR+TPMPA increased the NMDAR EPSC in the ON response 9-fold, in the OFF response 5-fold. Compared to glycine feedback, combined GABA feedback produces a similar suppression of the ON NMDAR EPSC but twice the suppression in the OFF pathway. The results confirm that GABA_C inhibition can regulate activation of NMDARs.

**Stimulus duration differentially activates NMDARs**

The pharmacological manipulations may reveal NMDAR pathways that are utilized by the retina or they may be a physiologically irrelevant epiphenomenon. We were able to differentially regulate NMDAR activation in ON-OFF cells by simply lengthening the light stimulus duration, thus providing evidence that NMDAR regulation by presynaptic inhibition occurs under physiological conditions.

The duration of the light stimulus was varied from 1 to 2 and 3 seconds under control conditions. In the OFF response, the 1s L-EPSC was significantly smaller than the 2s, which was similar to the 3s L-EPSC (Table 1, Fig 5A, B inset). The larger response after 2s probably results from photoreceptor adaptation. In two cells, the stimuli were increased to 4 and 5 seconds but this did not further increase the L-EPSC (data not shown). As expected, the ON L-EPSCs were similar in all three stimuli (Table 1, Fig 5A, B inset). The ratio of excitation (OFF L-EPSC/ ON L-EPSC) increased from 0.62 in 1s to 0.99 in 2s and 1.04 in 3s stimuli (Table 1, Fig 5B). Thus, the total charge of the OFF EPSC and the OFF/ON excitation ratio increased as the stimulus duration increased from 1 to 2 seconds and there was little change with further increase in stimulus duration.

To study the NMDAR EPSC in these responses, we selected neurons in which the 2s OFF L-EPSC was at least 50% larger than the 1s OFF L-EPSC. In these cells, the 2s and 3s OFF L-EPSCs were ~ 90% higher than the 1s OFF L-EPSCs (Table 1). D-AP5 (50 µM) reduced the OFF L-EPSCs for all three stimulus durations (Table 1, Fig 5A). The NMDAR component of the 1s OFF L-EPSC was 19% and increased to 36% with a 2s stimulus and to
27% with a 3s stimulus (Fig 5C). In the two cells in which the stimulus was increased to 4 and 5 seconds the NMDAR component was ~35%. Compared to the 1s OFF L-EPSC, the 2s OFF L-EPSC had a 3.6 fold increase in NMDAR EPSC; the 3s OFF L-EPSC had a 2.7 fold increase. Therefore, prolonging the stimulus from 1s to 2s increased the OFF EPSC, the total NMDAR charge, and the NMDAR fraction of the total EPSC.

The ON pathway regulates the NMDAR activation of the OFF pathway.

The ON pathway was blocked by L-AP4 to test whether NMDAR recruitment was due to interaction between ON and OFF pathways, (Slaughter and Miller 1981). D-AP5 was used to determine the amount of the EPSC that was due to NMDAR activation (Fig 6A). AP4 (20µM) in Mg$^{2+}$ free Ringer’s solution blocked the ON L-EPSC of transient cells completely and increased the 1 second OFF L-EPSC compared to control (223± 40%) (Fig 6A, B). D-AP5 (50 µM) reduced the AP4-enhanced OFF EPSC to 51 ± 5% (Fig 6A, B).

Thus, when the ON response was blocked the NMDAR component in the OFF response was significantly enhanced. Compared to control, where the ON response was present, there was a 2.6 fold increase in the OFF NMDAR component (from 19% to 49%) of the EPSC and a 5.8-fold increase in total NMDAR EPSC charge. The AMPA/KAR EPSC did not show a significant increase in L-AP4 (1.1 fold increase compared to control, p = 0.31, unpaired t-test). Thus, there are endogenous circuits between the ON and OFF pathways that selectively control the level of NMDAR activation. When this cross-talk is blocked, then the OFF response increases due to recruitment of NMDAR activation.

Blocking the ON pathway with AP4 augmented the NMDAR OFF component in a 1s stimulus (Fig 6). However, when a 2s stimulus was used, the NMDAR component in control was 36% of the total EPSC, and this was not significantly increased when crosstalk was blocked with AP4 (42%) (Table 1, Fig 6C). These results correlate with the experiments prolonging the light stimulus (Fig 5), where the OFF NMDAR EPSC was augmented in a 2s stimulus under control conditions. Thus, the OFF NMDAR-component was relatively small during a 1s stimulus and AP4 enhanced this component. But the OFF NMDAR-component was larger during a 2s stimulus and AP4 produced little enhancement.
This suggests that suppression of NMDAR pathways was due to short term crosstalk from the ON pathway and this crosstalk diminished with a 2s stimulus.

To evaluate involvement of GABA and glycine inhibition in ON-OFF crosstalk, the effects of L-AP4 on the OFF L-EPSC were tested after pre-treating the tissue with PTX+STR. In our experiments, we found that both gap junctions and inhibition influenced the effects of AP4. For the purpose of this study, we performed experiments in the presence of gap junction blockers in order to focus on the impact of cross inhibition. To block gap junctions, the tissue was pre-treated for 10 minutes with either 50 µM αGA (18α-glycyrrhetinic acid) or 100 µM MFA (meclofenamic acid). The effects of both gap junction blockers were similar and hence data were pooled for statistical analysis. L-AP4 increased the 1s OFF L-EPSC by 212 ± 23% in the presence of αGA/MFA (Fig 6E). D-AP5 reduced the enhanced L-EPSC to 41 ± 5%. Hence, the effects of L-AP4 and D-AP5 were similar in control (Fig 6B) and in αGA/MFA (Fig 6E) indicating that gap junction blockers did not alter the drug responses. The application of PTX and STR (in the presence of αGA/MFA) enhanced both the ON and OFF responses. But now the effect of L-AP4 was occluded (94 ± 7%) (Fig 6D, F). Thus ionotropic GABAergic and glycinergic crosstalk from the ON pathway regulates the NMDAR component in the OFF pathway. This illustrates an endogenous inhibitory circuit that regulates activation of NMDA receptor pathways. If gap junctions were active but inhibition was blocked, AP4 also increased the 1s OFF response. This second pathway through gap junctions was not explored.

**DISCUSSION**

This study demonstrates the prominent role that presynaptic inhibition plays in the control of NMDAR activation in retina. More importantly it illustrates that this regulation is dynamic and employed in normal visual function. In summary: 1) NMDAR activation is minimal when presynaptic inhibition is active, 2) glycinergic inhibition strongly suppresses NMDAR activation during the ON L-EPSC in ganglion cells, 3) concatenated synapses between GABA_A and GABA_C pathways reduce NMDAR current in both the ON and OFF L-EPSCs, and 4) short term crosstalk from ON to OFF pathways inhibits NMDAR activation in the OFF EPSC.
Background:

The role of NMDARs in retina has been paradoxical. In early studies comparing NMDA and KA effects on third order neurons in rabbit and salamander retina, both agonists produced strong depolarizations. Yet blocking NMDARs had little effect on light-evoked EPSPs (Bloomfield and Dowling 1985; Massey and Miller 1990; Slaughter and Miller 1983). Subsequent studies in amphibian retina demonstrated synaptic NMDAR responses but these experiments were performed in the presence of strychnine and picrotoxin (Diamond and Copenhagen 1993; Mittman et al. 1990). Still later it was shown that blocking inhibition allowed for spillover of glutamate, leading to activation of perisynaptic NMDARs in rodent retina (Sagdullaev et al. 2006; Zhang and Diamond 2006). The conclusion was that NMDARs were activated under conditions of excessive bipolar cell transmitter release and the implication was that this might not occur in the normal functioning of the retina.

A few specific populations of ganglion cells receive large synaptic NMDAR EPSCs, particularly OFF cells. For example, OFF alpha ganglion cells in the guinea pig retina have large NMDAR conductances, but ON alpha cells do not (Manookin et al. 2010). Similarly, OFF brisk sustained ganglion cells, but not ON brisk sustained, are driven largely by NMDAR activation in rabbit retina (Buldyrev et al. 2012; Buldyrev and Taylor 2013). In mouse retina, OFF cells have a larger NMDAR component compared to ON cells (Yang et al. 2011). However, the ON alpha cells in mouse have significant NMDAR conductances (Manookin et al. 2010).

The objective of our study was to evaluate the influence of GABAergic and glycinergic pathways in the control of light-driven NMDAR current in amphibian ganglion cells. It was somewhat surprising that feedback inhibition of bipolar cells completely reversed the relative dominance of AMPA and NMDA receptor currents at the ganglion cell. The implication is that a reduction in local feedback inhibition would be sufficient to augment NMDARs at particular synapses and this could change the EPSC dynamics. We could not determine if the small NMDAR current observed under our control experimental
stimulus conditions represented synapses that were disinhibited, rather than unregulated, constitutive NMDAR circuits.

Crosstalk regulates NMDARs in OFF pathway.

One circuit in which NMDAR activation is strongly regulated is the crosstalk between ON and OFF pathways. The OFF bipolar cell output to third order neurons is enhanced when the light stimulus is prolonged or when the ON bipolar light response is blocked. The enhanced OFF response was almost entirely due to recruitment of NMDARs. The enhanced OFF bipolar cell output may lead to spillover, but that response is part of the natural physiological repertoire of the retina.

Crosstalk between ON and OFF pathways is a prominent feature of visual processing and takes a number of forms. In crossover inhibition, excitation of the ON pathway inhibits the OFF pathway and vice versa. Crossover inhibition has been shown to be due to glycinergic amacrine cells in rabbit retina (Hsueh et al. 2008; Molnar and Werblin 2007). This type of crosstalk enhances the OFF response because it now represents both excitation and disinhibition. It functions to improve linearity (Molnar et al. 2009). L-AP4 blocks the crossover inhibitory pathway by blocking the ON pathway (Hsueh et al. 2008; Zaghloul et al. 2003). However, we find that L-AP4 enhances the OFF response, suggesting a different mechanism of crosstalk. This has been reported previously in the mudpuppy retina (Arkin and Miller 1988; 1987). The effect of L-AP4 on presynaptic mGluRs in bipolar terminals can also be ruled out as it would reduce the L-EPSC, opposite to our results (Awatramani and Slaughter 2001). Although we have determined that this crosstalk is through ionotropic inhibitory circuits, the mechanism by which it regulates the OFF L-EPSC is yet to be deciphered.

A similar crosstalk may be present in the OFF brisk sustained ganglion cells in rabbit retina (Buldyrev et al. 2012). The ganglion cells receive bipolar input that activates both AMPA and NMDA receptors and is regulated by glycinergic input from the ON pathway, both presynaptically to bipolar cell terminals and postsynaptically to the ganglion
cells. However, there is no evidence that glycine inhibition alters the balance of NMDA and
AMPA/KA activation in the pathway.

Crosstalk can also be excitatory. In amphibian retinal ON-OFF ganglion cells there is a small population in which the ON response is suppressed by GABA/glycine receptor antagonists, and a larger group in which these antagonists block the OFF response (Pang et al. 2007). It is postulated that this is indirect inhibitory input to one set of bipolars, from the opposing set, is mediated by inhibitory amacrine cells. We have not encountered examples of this type of crosstalk.

NMDAR activation is disproportionately regulated by presynaptic inhibition in both ON and OFF synapses.

We confirmed previous studies that NMDARs are expressed in third order neurons (Bloomfield and Dowling 1985; Kalbaugh et al. 2009; Mittman et al. 1990; Zhang and Diamond 2006; 2009) but their activation is minimal when presynaptic inhibitory circuits are intact (Coleman and Miller 1988; 1989; Slaughter and Miller 1983). Several studies found synaptic NMDARs only in the OFF pathway (Sagdullaev et al. 2006; Zhang and Diamond 2009). This might explain why the NMDAR component, although small, was significant only in the OFF L-EPSC under our control conditions. Amacrine cells also possess NMDARs and AMPA/KARs (Dixon and Copenhagen 1992). This could mean that NMDAR antagonists would reduce amacrine cell excitation and consequently reduce feedback inhibition to bipolar cells. The reduced inhibitory feedback could potentially enhance NMDAR activation on ganglion cells but this would be blocked in our experiments because of the NMDAR antagonist.

When inhibitory circuits are blocked then NMDARs contribute significantly to light responses in ON-OFF transient cells (Diamond and Copenhagen 1993; 1995; Mittman et al. 1990; Taylor et al. 1995). However, the extent of this transition had not been characterized previously. Hence our observations bridge the varied literature in the long-standing debate on whether NMDARs contribute to light responses of retinal ganglion cells. The sheer magnitude of the total increase in ON pathway synaptic (4X) and NMDAR EPSCs (35X)
regulated by presynaptic inhibition is noteworthy. When comparing this to the 1.7X increase in AMPA/KAR EPSCs, it is clear that NMDAR activation is disproportionately regulated by presynaptic inhibition. The supposition is that AMPA/KARs are located at the synapse and saturated by glutamate release. Therefore, increases in AMPA/KAR currents represent recruitment of additional synapses. On the other hand, NMDARs may be largely peri-synaptic and can be stimulated by the spillover that results from more glutamate release at the active sites (Chen and Diamond 2002; Sagdullaev et al. 2006) (Fig 7). Most of the studies on the relative strength of activation of NMDARs and AMPA/KARs in the past have blocked inhibitory circuits in order to isolate excitation (Chen and Diamond 2002; Kalbaugh et al. 2009; Matsui et al. 1998; Sagdullaev et al. 2006). Our observations suggest that these protocols not only simplify the retinal network but fundamentally alter the properties of excitatory signaling.

GABA and Glycine inhibition.

Glycinergic amacrine cells in mammalian retina are generally narrow field (Menger et al. 1998; Pourcho and Goebel 1987) and form approximately 50% of the amacrine cell population (Koontz et al. 1993; MacNeil and Masland 1998; Wassle et al. 1986). In salamander retina there are multistratified widefield and narrowfield glycine neurons (Yang et al. 1991) and glycine input is found at both the synaptic axon terminal and more distally at bipolar cell dendrites (Maple and Wu 1998). The axon terminal input is from amacrine cells, the dendritic input from interplexiform cells (Shen and Jiang 2007). We found that GlyRs exerted more control in directly regulating NMDAR activation than either GABA\textsubscript{C}Rs or GABA\textsubscript{A}Rs. This may be the result of amphibian bipolar cells receiving dual glycine input. But glycine input from OFF-responding interplexiform cells was found to inhibit ON bipolar cells in the dark (Maple and Wu 1998). Thus ON signals would get opposing glycine signals at light onset: disinhibition from OFF interplexiform cells and inhibition from ON and ON-OFF amacrine cells.

Signals from GABA receptors were only found at the axon terminal of amphibian bipolar cells (Maple and Wu 1996). Mouse rod bipolar terminals express GABA\textsubscript{A}, GABA\textsubscript{C}
and glycine receptors, although GABA<sub>C</sub>-R currents dominate (Eggers and Lukasiewicz 2006) and GABA<sub>C</sub> inhibitory circuits regulate NMDAR activation in mouse (Matsui et al. 2001; Sagdullaev et al. 2011; Sagdullaev et al. 2006). Our findings in intact salamander retina are that NMDAR pathway regulation by GABA<sub>C</sub>-Rs only becomes prominent when GABA<sub>A</sub>R pathways are blocked (Fig 7).

**Serial inhibition indirectly regulates NMDAR activation through GABA<sub>C</sub>Rs.**

Serial inhibition, in which amacrine cells inhibit other amacrine cells, makes it difficult to fully evaluate the importance of the individual transmitter systems (Eggers and Lukasiewicz 2006; Eggers et al. 2007; Roska et al. 1998; Vigh et al. 2011; Zhang et al. 1997). This is most evident in our experiments for the GABAergic pathways in the intact retina, where selective suppression of either GABA<sub>A</sub>Rs or GABA<sub>C</sub>Rs had only a small effect on synaptic excitation. But when both receptors were blocked then the NMDAR total charge in the ON EPSC increased 9-fold. This suggests interdependence between the two receptors. Blocking only GABA<sub>A</sub>Rs made the ON L-EPSCs transient, indicating an increase in sustained inhibitory circuits, most likely GABA<sub>C</sub>R circuits. We did not observe reciprocal effects on GABA<sub>A</sub>R pathways when blocking GABA<sub>C</sub>Rs. Hence it is likely that GABA<sub>A</sub>Rs not only provide inhibition to bipolar terminals but also to amacrine cells activating GABA<sub>C</sub>Rs (Fig 7). A similar circuit has been described recently in the surround inhibition of rabbit retinal ganglion cells (Buldyrev and Taylor 2013). Sometimes even blocking GlyRs decreased the L-EPSCs indicating that GABAergic amacrine cells can be regulated by GlyR activation. This is evident in the ON NMDAR responses, where blocking glycine receptors increased the response 10-fold, blocking GABA receptors produced a 9-fold increase, but blocking all ionotropic GABA and glycine receptors increased the ON NMDAR total charge by 35-fold. The reason the individual receptor blockers do not add up to the total block is presumably the effects of serial inhibition. Therefore, it is likely that we are underestimating the magnitude of each transmitter system in regulation of NMDAR pathways.
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FIGURE LEGENDS:

Figure 1: Comparison of the NMDAR current in the light-evoked EPSCs (L-EPSCs) and the electrically-evoked EPSCs (E-EPSCs). A, shows the L-EPSCs for an ON-OFF transient cell under control, Mg$^{2+}$ free Ringer’s solution, and in Mg$^{2+}$ free with 50 µM D-AP5. The 1s light stimulus is represented by the bar at the top. The inset below the current traces shows the raster plot for spike activity evoked by a series of three 1s light stimuli. B, shows the quantification of the effect of Mg$^{2+}$ free Ringer’s solution alone or with D-AP5 compared to control (n = 13). Mg$^{2+}$ free Ringer’s solution had a small effect on the L-EPSCs (ON: p = 0.77, OFF: p = 0.47). D-AP5 also had a small effect on L-EPSCs, statistically significant only in the OFF pathway (ON: p = 0.44, OFF: *p < 0.05). C, shows the electrically stimulated EPSCs (E-EPSCs) of a cell in the ganglion cell layer of a slice preparation in control, in Mg$^{2+}$ free Ringer’s solution alone and with and D-AP5. The stimulus was a 1 ms pulse applied to the outer plexiform layer. D, shows the quantification of the effect of Mg$^{2+}$ free Ringer’s solution and D-AP5 compared to control (n = 4). Mg$^{2+}$ free Ringer’s solution greatly enhanced the E-EPSCs (*p < 0.05) and D-AP5 blocked a large portion of the E-EPSC (#p ≤ 0.005).

Figure 2: L-EPSCs had a large NMDAR component when presynaptic inhibition is blocked. A, shows the ON-OFF L-EPSCs of a cell in control, 100 µM PTX+ 10 µM STR in Mg$^{2+}$ free Ringer’s solution alone or with 50 µM D-AP5. B, shows the quantification of the effects of PTX+STR and D-AP5+PTX+STR in Mg$^{2+}$ free Ringer’s solution compared to control (n = 6). PTX+STR greatly enhanced the L-EPSCs (ON: *p < 0.01, OFF: **p < 0.005) and D-AP5 blocked a large portion of the enhancement (ON: #p < 0.001, OFF: #p < 0.001). C, shows the comparisons of the NMDAR component in PTX+STR vs control in the ON and OFF pathways (#p < 0.001, unpaired t-test). D, shows the ON-OFF L-EPSCs of a cell in D-AP5 alone and then with the addition of PTX+STR. E, shows the change in the total control L-EPSC produced by D-AP5 alone and by D-AP5+PTX+STR in both ON and OFF pathways (n = 6, ON: p = 0.12, *OFF: p ≤ 0.05). F, shows the ON-OFF L-EPSCs of a cell in control, then in PTX+STR in Mg$^{2+}$ free Ringer’s solution, and then after the addition of 50 µM D-AP5+ 10 µM NBQX to the PTX+STR+ Mg$^{2+}$ free Ringer’s solution.
Figure 3: Effects of GABA and glycine antagonists on the NMDAR current. A, shows the L-EPSCs of a cell in Mg\(^{2+}\) free Ringer’s solution, then with addition of 10 µM STR, and then with 50 µM D-AP5 and STR in Mg\(^{2+}\) free Ringer’s solution. B, shows the quantification of the effects of STR alone and D-AP5 + STR compared to Mg\(^{2+}\) free Ringer’s solution (n = 6). STR enhanced the L-EPSCs (ON: *p < 0.05, OFF: p = 0.06) and D-AP5 blocked a significant portion of the enhanced L-EPSC (ON: #p < 0.005, OFF: #p < 0.005). C, shows the L-EPSCs of a cell in Mg\(^{2+}\) free Ringer’s solution, then plus SR95531, then plus SR and D-AP5. D, shows the quantification of the effects of SR alone or of D-AP5 + SR, compared to Mg\(^{2+}\) free Ringer’s solution (n = 7). The effects on both the total and peak L-EPSCs are shown. SR produced a statistically significant increase in total synaptic charge only in the OFF L-EPSC (ON: p = 0.75, OFF: *p < 0.05). D-AP5 significantly reduced only the OFF L-EPSC (ON: p = 0.1, OFF: *p < 0.05). SR increased the peaks of the L-EPSC (ON: +p ≤ 0.01, OFF: +p ≤ 0.01) while D-AP5 significantly reduced only the enhanced OFF peak (ON: p = 0.075, OFF: *p < 0.05). E, shows the L-EPSCs of a cell in Mg\(^{2+}\) free Ringer’s solution, then plus 100 µM TPMPA, and then plus TPMPA and D-AP5. F, shows the quantification of the effects of TPMPA, and D-AP5 in TPMPA, compared to Mg\(^{2+}\) free Ringer’s solution (n = 7, 8). TPMPA enhanced the L-EPSCs (ON: **p < 0.001, OFF: *p < 0.05) but D-AP5 did not reduce the enhanced L-EPSCs significantly (ON: p = 0.13, OFF: p = 0.41). G, H, show the comparison of NMDAR components in STR, SR, and TPMPA compared to control or PTX+STR in the ON and OFF pathways respectively. In the ON pathway, only STR had a NMDAR component significantly higher than control (STR: #p < 0.005, SR: p = 0.67, TPMPA = 0.6, unpaired t-tests). In the OFF pathway, none of the three antagonists had a significantly higher NMDAR component than control (STR: p = 0.34, SR: p = 0.37, TPMPA = 0.36, unpaired t-tests).

Figure 4: GABACRs suppressed a significant NMDAR component in L-EPSCs in the retinal slice. In the retinal wholemount, GABACRs suppressed a large NMDAR component only when GABA\(_A\)R inhibition was also blocked. A, shows the L-EPSCs of a cell in a retinal slice in Mg\(^{2+}\) free Ringer’s solution, plus 100 µM TPMPA, then 50 µM D-AP5 in
TPMPA. B, shows the quantification of the effects of TPMPA and of D-AP5+TPMPA compared to Mg$^{2+}$ free Ringer’s solution (n = 6). TPMPA enhanced the L-EPSCs (ON: *p \leq 0.01, OFF: *p < 0.01) and D-AP5 reduced the enhanced L-EPSCs significantly (ON: *p \leq 0.01, OFF: #p < 0.005). C, shows the comparison of the NMDAR component of the TPMPA-enhanced L-EPSCs in retinal slices and wholemounts. This component was significantly higher in retinal slices compared to wholemounts (ON: *p < 0.05, OFF: #p < 0.005, unpaired t-tests). D, shows the L-EPSCs of a cell in the retinal wholemount in Mg$^{2+}$ free, SR+TPMPA then D-AP5 in SR+TPMPA. E, shows the quantification in the wholemount retina of the effects of SR+TPMPA vs. D-AP5+SR+TPMPA compared to Mg$^{2+}$ free Ringer’s solution (n = 5). SR+TPMPA increased the L-EPSCs (ON: *p < 0.05, OFF: *p < 0.05) and D-AP5 reduced the enhanced L-EPSCs significantly (ON: *p < 0.05, OFF: #p < 0.005). F, shows the comparison of the NMDAR component of L-EPSC in the wholemount retina in SR+TPMPA compared to control. SR+TPMPA had a higher NMDAR component than control (ON: p = 0.09, OFF: *p < 0.01, unpaired t-tests).

Figure 5: NMDAR component of the OFF L-EPSC increased with stimulus duration. A, shows the L-EPSCs of a transient ON-OFF cell to a series of light stimuli of 1, 2 and 3s duration in Mg$^{2+}$ free Ringer’s solution and then with 50 µM D-AP5 added. B, shows the ratio of OFF/ON L-EPSC for these stimuli (n = 17, 18). The ratio is significantly higher for 2 and 3 second stimuli compared to the 1 second stimulus (2s: *p \leq 0.01, 3s: *p < 0.01). The inset shows the 2 and 3 second L-EPSCs compared to the 1 second ON and OFF L-EPSCs. The 2 and 3 second stimuli have a significantly higher OFF L-EPSC compared to the 1 second stimulus (table 1, #p < 0.001). However the ON L-EPSC did not change significantly (table 1). C, shows the comparison of the NMDAR component in the OFF L-EPSC for the three stimuli (n = 7). The NMDAR components in the 2 and 3s stimuli were significantly higher than the 1s stimulus (table, 2s: **p \leq 0.01, 3s: *p < 0.05).

Figure 6: Blocking crosstalk increased the NMDAR component of the OFF L-EPSC. A, shows a cell’s L-EPSCs to a 1s light stimulus in control, then with 20 µM L-AP4 in Mg$^{2+}$ free Ringer’s solution, then with the addition of 50 µM D-AP5 for 1 second stimuli. B, shows the mean total OFF L-EPSC produced by a 1s light stimulus, normalized to the OFF
response in control (n = 8). L-AP4 in Mg$^{2+}$ free Ringer’s solution blocked the ON L-EPSC completely and increased the OFF L-EPSC significantly (*p < 0.05). D-AP5 reduced a large fraction of the enhanced OFF L-EPSC ("p < 0.005). C, shows the comparison of the NMDAR component of the OFF L-EPSC in control and L-AP4 for 1 and 2s stimuli. The NMDAR component of the OFF response in the presence of L-AP4 was not significantly different between 1 and 2s stimuli (p = 0.29). The NMDAR component of the 1 second OFF L-EPSC was significantly greater in L-AP4 ("p < 0.005, unpaired t-test). However, the NMDAR component of the 2s OFF L-EPSC was not significantly different (p = 0.49, unpaired t-test). D, shows L-EPSCs of a cell in PTX+STR in Mg$^{2+}$ free Ringer’s solution containing 50µM 18-αGA, and then with the addition of L-AP4 for 1s stimuli. E, shows the effect of L-AP4 and D-AP5 on the relative mean total OFF L-EPSC in Mg$^{2+}$ free Ringer’s solution containing either 18-αGA or 100 µM MFA. L-AP4 increased the OFF EPSC (*p< 0.005; n = 8), while D-AP5 blocked a large component of the enhanced EPSC ("p < 0.001; n = 5). F, shows the normalized total OFF L-EPSC in PTX+STR (in Mg$^{2+}$ free Ringer’s solution containing 18-αGA/MFA) and the effect of L-AP4. L-AP4 did not increase the OFF-LEPSC in the presence of PTX+STR (p = 0.42; n = 7).

Figure 7: Proposed model of inhibitory feedback to bipolar cells that regulates their glutamate release and activation of ganglion cell excitatory receptors. RGCs express AMPA/KARs and NMDARs in the synaptic and peri-synaptic space, respectively. The activation of NMDARs is determined by enhanced glutamate release from bipolar terminals, regulated by inhibition from glycinergic and GABAergic amacrine cells. The GABA$\alpha$R pathway acts to both inhibit bipolar cells and inhibit GABA$\gamma$R pathways. Under the control conditions depicted in the figure, AMPA/KARs, but few NMDARs, are activated. GABA$\gamma$Rs feedback is low because of GABA$\alpha$R inhibition. Block of glycinergic inhibition or both GABA$\alpha$R and GABA$\gamma$R inhibition augments glutamate release and consequently perisynaptic NMDARs become activated.
Table 1 shows a comparison of the ON and OFF L-EPSCs for 1, 2 and 3 s light stimuli in control, 50 μM D-AP5, and 20 μM L-AP4 and D-AP5.

Data Rows 1 and 2: The OFF response to 2s or 3s stimulation is larger than to 1s stimulation.

Data Rows 3-5: This tabulates a subpopulation of the cells in the first row in which the OFF response to a 2s light stimulus is at least 50% larger than the OFF response to a 1s light stimulus. D-AP5 suppressed the 1s, 2s and 3s OFF responses compared to the control subset (p ≤ 0.01). When the ON response was blocked by L-AP4, D-AP5 suppressed the 1s and 2s OFF response (p < 0.001).

All responses are normalized to the control ON and OFF EPSCs for a 1s light stimulus.

Table 1

<table>
<thead>
<tr>
<th>Recording condition</th>
<th>1 second light</th>
<th>2 second light</th>
<th>3 second light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ON</td>
<td>OFF</td>
<td>ON</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td>Control subset</td>
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<td>100</td>
<td>92</td>
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<tr>
<td>D-AP5 on subset</td>
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<td>9%</td>
<td>81</td>
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<tr>
<td>D-AP5 on L-AP4 treated subset</td>
<td>-</td>
<td>51</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig 1

A. 1 s Light Stimulation

B. % total L-EPSC

C. 1 ms Electrical Stimulation

D. % total E-EPSC

Legend:
- control
- Mgfree
- +AP5

Graph B shows the % total L-EPSC with light stimulus ON and OFF.

Graph D shows the % total E-EPSC with electrical stimulus.
Fig 4

A (Slice) 1s light
200 pA Mgfree +TPMPA +TPMPA+AP5

D (Whole mount) 1s light
100 pA Mgfree +SR+TPMPA +SR+TPMPA+AP5

B % total L-EPSC
ON OFF
\[
\begin{align*}
\text{Mgfree} & & \ast \ast \\
\text{+TPMPA} & & \\
\text{+TPMPA+AP5} & \\
\end{align*}
\]

C % NMDAR component
ON EPSC OFF EPSC
\[
\begin{align*}
\text{whole mount} & & \\
\text{slice} & & \\
\end{align*}
\]

E % total L-EPSC
ON OFF
\[
\begin{align*}
\text{Mgfree} & & \ast \ast \\
\text{+SR+TPMPA} & & \\
\text{+SR+TPMPA+AP5} & & \\
\end{align*}
\]

F % NMDAR component
ON EPSC OFF EPSC
\[
\begin{align*}
\text{control} & & \\
\text{SR+TPMPA} & & \\
\text{control} & & \\
\text{SR+TPMPA} & & \\
\end{align*}
\]

Legend:
- \ast: Significant difference
- \#: Significant difference compared to control
Fig 5
Figure 7